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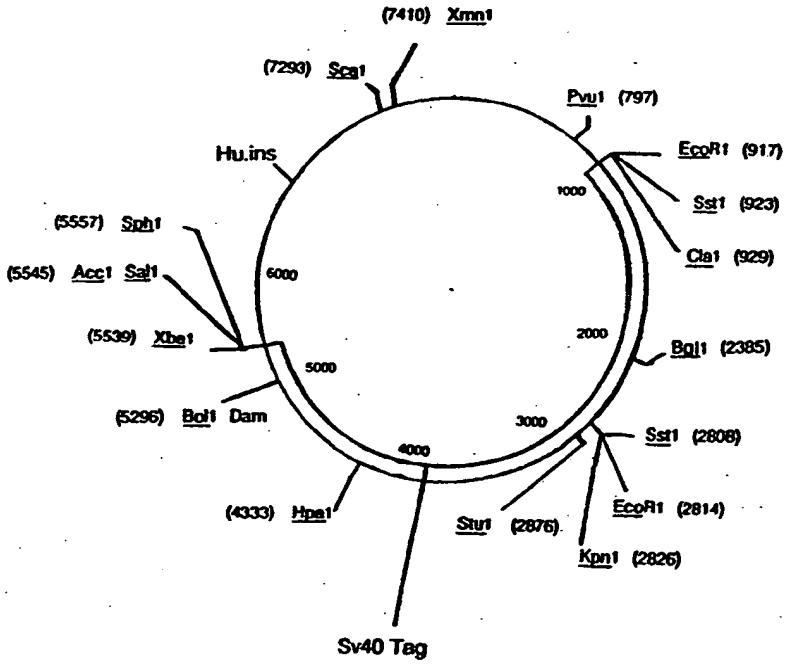
(54) Title: TRANSFORMED CELL LINES



(57) Abstract

Cells capable of expressing a desired polypeptide have been modified to include an oncogene under the control of the promoter for the polypeptide gene. For example, pancreatic β islet cells may be modified to produce controllable levels of insulin.

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TRANSFORMED CELL LINES

Field of the Invention

This invention relates to transformed (and immortalised) cell lines and to their preparation.

Background of the Invention

There are many proteins of the body which have a very restricted expression. Such proteins include haemoglobin (only found in red blood cells), keratin (only found in skin) and insulin (only found in the ß islet cells of the pancreas). Advances in molecular biology have made it possible to clone the genes for many of these important biological molecules, and they can thus be produced in large amounts by bacteria or yeast growing in fermenting vessels. While this approach has been an important advance for the study of these molecules and their use in clinical treatment, there is still much to learn about the physiology of the cells which produce them and how the regulation of gene expression is controlled. Many products of specialised cells remain to be defined, owing to a lack of sufficient suitable cells.

It is therefore desirable to produce cell lines which can be grown up in large numbers using tissue culture techniques. It seems likely that the growth of most cells is controlled by a complex "balancing act" of stimulating and inhibitory growth factors which act at very close range in picogram amounts. The factors which control the growth and differentiation of leukocytes are under intensive investigation and many cytokines (e.g. the interleukins and colony-stimulating factors) have been cloned. The purified cytokines can be used to grow specific cells in culture (e.g. IL2 receptor-positive lymphocytes) but, for most cell types, the molecules involved in stimulating cell growth are completely unknown. For such cells, the most common method for deriving cell lines is to culture tumours which have formed in vivo. These can be either naturally-occurring tumours or those induced by mutagenic techniques, e.g. irradiation or chemicals. However, many of these

tumour cells do not behave in vitro in the same way that the parent cells behave in vivo. For instance, two commonly-used pancreatic ß-cell tumour lines, derived from the rat and hamster, RIN 5F and HIT, produce very little insulin and have a minimal response to glucose.

A recent advance in the study of tissue-specific gene expression has been in the use of transgenic mice. A cloned gene introduced into a fertilised egg can be expressed, in a genetically-inheritable fashion, in the offspring. Hanahan, Nature 315 (1985) 115, reports that SV40 (large T antigen), when expressed in ß islet cells of transgenic mice, can cause ß islet cell tumours. Teitelman et al, Cell 52 (1988) 97, report that mouse ß islet cell lines which grew in vitro could be derived from some of these tumours, albeit with some difficulty.

Immortalised monoclonal antibody-producing cell lines are prepared, by the classical Milstein technique, by reaction between myeloma cells and cells having the desired function, and careful selection of the large variety of fusion products. The exact content of the hybridomas is unclear, since they are selected functionally rather than being defined structurally. The technique is suitable for preparing rat and mouse cell lines, but has generally proved unsuitable as a means to obtaining human monoclonal antibodies.

Epstein-Barr virus cells have been used to transform human B lymphocytes, and to give a human IgM monoclonal-producing cell line. However, this function is short-lived.

The human insulin gene itself has been cloned and can be expressed in bacteria and yeast, but there is much that is not understood about the structure of the ß islet cells and the way insulin release is controlled. Most in vitro research is performed on either the HIT (hamster-derived) or RIN (rat-derived) ß islet cell lines. These produce only low levels of insulin in response to glucose. Insulinomas have been produced in vivo in transgenic mice

after transfer of the SV40 large T antigen in association with the rat insulin gene (Hanahan, supra). However, these cells have been hard to establish in vitro and are, in any case, mouse ß cell lines.

This invention has two particular objects, one being to produce cell lines, which can be cultured long-term (and perhaps indefinitely) using standard tissue culture technology, from primary tissues that still retain the biological characteristics of the original primary tissue at least in part, and the other to produce human monoclonal antibodies or other biological products.

Summary of the Invention

Cells according to the present invention, that are capable of expressing a desired polypeptide, have been modified to include an oncogene (or more than one oncogene) under the control of a promoter that is heterologous with respect to the oncogene. The promoter may be tissue-specific, an inducible or selectable promoter, or the cells' own promoter for the polypeptide gene. The oncogene's promoter will usually be absent or inactivated.

Such cells may be prepared by transformation with a plasmid comprising the oncogene(s) under the control of the tissue-specific genetic expression elements.

The recombinant DNA that is used constitutes another aspect of the invention. The DNA may be in the form of a plasmid.

Description of the Drawings

Figure 1 shows a plasmid embodying the invention.

Figure 2 is a graph showing insulin production for an embodiment of the invention.

Description of the Invention

The accompanying Table provides a partial list of types of cells to which the present invention can be applied, the appropriate promoter and possible uses.

In one embodiment of the invention, the cells, e.g. ß islet cells, are normally incapable of expressing the desired polypeptide, e.g. insulin, during growth.

Therefore it is thought that stopping proliferation of cells may be essential to allow them to express their differentiated functions. The presence of the oncogene allows growth, in order to obtain a satisfactory number of cells, but it may be desirable to switch off the oncogene(s) when it is desired to produce, say, insulin in response to glucose. Switching-off may be achieved by the presence of an anti-sense (with respect to coding) oligonucleotide in the cells.

Anti-sense oligonucleotides or cDNA are a sequence of nucleotides which is complementary to that of the protein-encoding region (the sense sequence) of a gene. The anti-sense sequence when combined with the sense mRNA hybridises and results in the prevention of translation of the mRNA into protein. The anti-sense sequence may be introduced into a cell either as the addition externally of short oligodeoxynucleotides (or chemical derivatives thereof) which enter the cell by pinocytosis or other mechanisms, or by the aid of chemical or physical means. Alternatively, the modification comprises cloring in DNA encoding a suitable anti-sense oligonucleotide and an associated promoter which can be switched on and off by an external stimulus.

Temperature-sensitive (TS) mutant oncogenes may be particularly useful for switching cell growth on and off. In an illustrative embodiment, the oncogene will only function at certain temperatures, for SV40TS mutant, at about 33°C, while at elevated temperatures, c. 40-41°C for SV40 TS, the oncogene no longer functions, and so the cells stop growing. Other methods of stopping growth include irradiation of the cells and the use of drugs.

To permit more rapid selection of transfected cells, putting a selectable marker gene, e.g. for resistance to neomycin (neo^R), under the control of a cell-specific promoter (such as the insulin promoter) permits more rapid removal of contaminating cells such as fibroblasts. For example, the insulin-promoter-neo^R construct (i.e. insulin

promoter + sequence conferring resistance to neomycin) and the insulin promoter-oncogene construct could be linked or co-transfected. After transfection, a drug toxic to cells, but destroyed by neo^R, e.g. G418, may be used to remove untransfected cells, or cells of the wrong lineage from the population. Another variant is to use a non-tissue-specific promoter to drive the production of the oncogene and the selectable marker, especially those which are inducible. These include HLA and metallothionin, respectively inducible by interferon and by heavy metals such as zinc. These systems may be useful in conditions where the cells are reasonably pure, but do not grow adequately, e.g. T lymphocytes.

Transformed islet cells according to the invention, having the capability of producing insulin in response to glucose, may be used in the form of a therapeutic implant, as an artificial pancreas. In order to prepare a suitable implant, known encapsulation technology may be employed.

Modified B lymphocytes according to the present invention can produce human monoclonal antibodies. For example, plasmid constructs may be made which will express oncogene or combinations of oncogenes, the expression of which is controlled by immunoglobulin gene-specific elements or other B lymphocyte-specific promoters or non-cell-specific inducible promoters (as illustrated above).

A specific example of a plasmid including the human insulin gene (Hu.ins) and the SV40 gene is shown in Figure 1 of the accompanying drawings. This plasmid is designated as ss ins Tag. Such constructs may be transfected into human ß islet cells by, for example, calcium phosphate precipitation or electroporation. Subsequent immortalised cell lines may be tested for the retention of the original biological functions in the absence or presence of anti-sense oligonucleotides given externally or activated internally.

Hitherto, it has been difficult to obtain human ß islet cells in large quantities. By means of the

invention, however, the ability to produce human cell lines of the type described above may have far-reaching consequences in biomedical research. In particular, the ability to grow insulin-producing human ß islet cells in culture may have a major impact in various areas of diabetic research. An in vitro system can be used to derive human ß islet cell lines (see below). These may be use as a source of antigen to define the molecules involved in the initiation and progression of the diabetic disease process utilising T lymphocyte clones and sera from newly diagnosed Type 1 diabetic patients. They may also be used for testing potential drugs for their use as enhancers of insulin release (e.g. clenbuterol). These would be very beneficial to the many Type I and Type II diabetic patients which have impaired insulin release (0.5 million in the U.K.). They may be used in the study of the molecules expressed by the ß islet cell which lead to its destruction in diabetes. This may permit interfering with the auto-immune state by, for example, inducing immunological tolerance, and may have many other uses.

As indicated by the Table, the invention can be used to derive various important cell lines. By way of example, the insulin gene enhancer and promoter elements which ensure expression of the SV40 large T antigen have been used in human ß islet cells, but it would also be possible to combine large T antigen expression with the expression of the Ha-ras Ki-ras or N-ras oncogenes or any one of a number of other oncogenes in various combinations. The plasmid construct (SVTag) could be used to promote SV40 large T antigen expression in other cell types, by inserting the appropriate tissue-specific promoting elements in place of insulin. For instance, thyroid cells could be transformed with SV40 large T controlled by thyroglobulin expression elements of the gene. As all cell types have their own repertoire of proteins, it should be possible to tailor SVTag derivatives or any other oncogene to transform the cell type of choice in vitro.

The transformation of, say, B lymphocytes according to the invention provides cell lines that can be selected for continuous production of human monoclonal antibodies of any immunoglobulin class, e.g. IgG, IgA or IgE, as well as IgM, and are therefore distinct from the majority of Epstein-Barr virus transformants.

The following Example illustrates the invention.

Example

Human Islets

Purified human islets are obtained by known digest methods (Lake et al (1989) Diabetes 38, 143-145; Ricordi et al (1989) Diabetes 37, 413-420). Briefly, human pancreata obtained with permission from organ donation are transported to the laboratory (approximately 50/year) with a minimal warm ischaemic time and a cold ischaemic time of less than 3 hours. After loading via the pancreatic duct with collagenase (Serva, 4 mg/ml, 2 ml/g pancreas), the pancreas is placed in a digesting chamber through which minimal essential medium (MEM) circulates until free islets are seen. The chamber is then shaken and the medium switched to an open circuit to collect the pancreatic digest which is washed with MEM. The digest is then separated on a large-scale discontinuous BSA gradient using the COBE 2991 cell separator, which yields a purified islet preparation. The islets obtained, represent only 1-2% of the tissue in the digest, and the yield varies from 10^5 to 5×10^5 islets (150 μm islet equivalents) per preparation, with a purity of 50 to 90%.

Islet Culture

Islets are cultured free-floating in sterile Petri dishes using RPMI medium containing 10% foetal calf serum.

After electroporation, the islets are transferred to tissue culture dishes (to enhance attachment and cell outgrowth) in the same medium.

Electroporation

Whole islets are exposed to a 1 second pulse of 300 V with a capacitance of 760 μF using a Progenitor II (PG2000) electroporator (Hoeffer Instruments) in the presence of purified ss ins Tag DNA (15 μg) and pTCF DNA (1 μg).

Plasmids

The known SV40 large T antigen promoted by the human insulin gene promoter sequence (ss ins Tag) is used. pTCF contains the neo^R sequence conferring resistance to the antibiotic G418.

Insulin Assay

This is done using ^{125}I-insulin (Amersham), guinea pig anti-insulin (Miles) and donkey anti-guinea pig-coated cellulose (Sac-cel, Wellcome). It accurately measures insulin below 1 ng/ml.

Results

The optimal conditions for electroporation using pTCF (as a selective marker) and mouse L cells were at a capacitance of 710 μF and a voltage of 410 V; an efficiency close to 1 in 10^3 transformants was achieved. This knowledge has been applied to work on islet cells which are more delicate. The conditions for islets have been redefined as 710 μF, 300 V.

Transfected cultures are monitored for the release of insulin, which is usually high at the beginning, gradually tailing off with culture time. Successful transfectants have the characteristics of increasing cell numbers (cell growth) and a rise in insulin release. This is shown in Figure 2 of the accompanying drawings, which is a graph of IP, i.e. insulin production per day (ng/ml) against days in culture (T).

Such cell lines have been established which have been cloned and passaged for greater than 50 generations. The cells produced so far (COBE 18.2 has been studied in the greatest detail) have insulin granules and produce insulin in the early subcultures.

On culture in various media, their growth rate changes and they can double every 24-36 hours in RPM1 1640 +10% FCS. Clones vary in morphology, and some are very epitheloid in appearance. For example, one such clone has been shown to have vesicles and granules, and expressed cytokeratins 8 and 18, typical markers of simple epithelial cells.

TARGET CELLS	PROMOTER	POTENTIAL USES INCLUDE:
T lymphocyte	e.g. α or β of T cell receptor λ or δ of T cell receptor CD3 of T cell receptor	Studies of T cell recognition, identification of new cytokines, cell surface protein
B lymphocytes	e.g. Ig promoter, H or L chain, B cell specific markers, e.g. CD19, CD40, B cell subset marker, e.g. CD5	Generation of monoclonal antibody, identification of new cytokines and cell surface proteins
Macrophages	e.g. c-fms (receptor for macrophage colony stimulating factor)	Study of antigen presentation identification of new macrophage products: cytokines, other products, receptors etc.
Dendritic cells	e.g. HLA class II	Study of antigen presentation, identification of new cytokines and cell surface receptors
Keratinocyte	e.g. keratin	Use for covering burns, ulcers
Endothelium	e.g. von Willebrand factor	Grafts, cytokines, vessel repairs
Islets of Langerhans	e.g Insulin, glutamic acid decarboxylase (64K protein)	Islet grafts in diabetes
Bone	e.g. bone morphogenic proteins	Grafts, new cytokines
Cartilage	e.g. collagen type II	Grafts, new cytokines
Mast cells	e.g. histidine decarboxylase	Identification and study of inflammatory mediators, cytokines
Neurons	e.g. dependent on type enzyme involved in neurotransmitter synthesis, cytokeratin, etc.	Grafts, study of transmitters and receptors
Megakaryocytes		Platelets, identification of platelet products
Liver	e.g. albumin promoter	Liver products, e.g. Factor VIII for haemophiliacs

CLAIMS

1. Cells capable of expressing a desired polypeptide, which have been modified to include an oncogene under the control of a promoter that is heterologous with respect to the oncogene.

2. Cells according to claim 1, which also include a selectable marker gene under the control of a cell-specific promoter.

3. Cells according to claim 2, in which the selectable marker is a drug-resistance gene.

4. Cells according to any preceding claim, in which the polypeptide is not expressed during growth, which have additionally been modified such that the oncogene can be switched on or off, thereby respectively allowing growth of the cells or production of the polypeptide.

5. Cells according to claim 4, which are pancreatic ß islet cells and wherein the polypeptide is insulin.

6. Cells according to claim 4 or claim 5, which include DNA encoding an anti-sense (with respect to coding) oligonucleotide under the control of its own promoter.

7. Cells according to any of claims 1 to 3, which are B lymphocytes capable of producing a human monoclonal antibody.

8. Cells according to any preceding claim, in which the oncogene's promoter is absent or inactivated.

9. Cells according to any preceding claim, in which the said heterologous promoter is tissue-specific or an inducible or selectable promoter.

10. Cells according to any of claims 1 to 8, in which the said heterologous promoter is the promoter for the polypeptide gene.

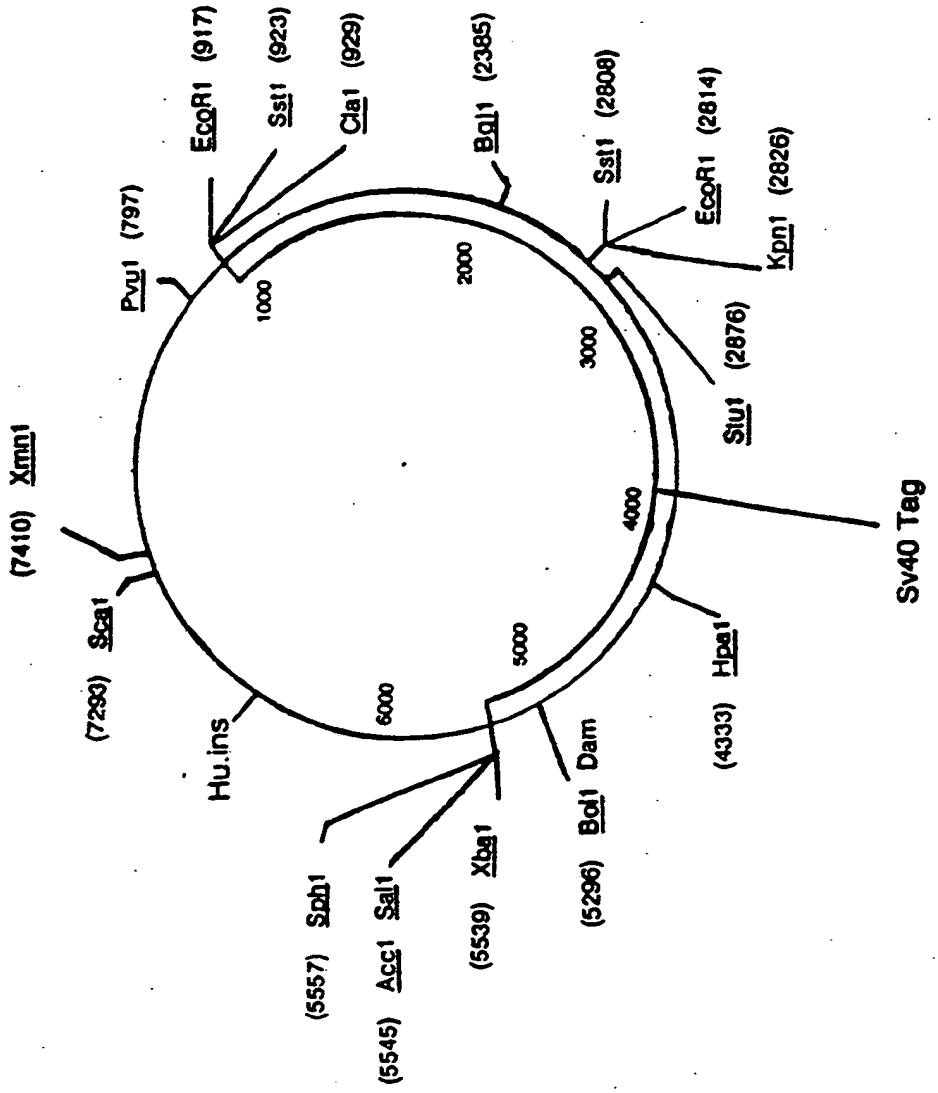
11. Cells according to any preceding claim, which are human cells.

12. Cells according to claim 10 and claim 11.

13. A human monoclonal antibody produced from cells according to claim 7.

14. Recombinant DNA as found in cells according to any of claims 1 to 12.

15. A plasmid comprising an oncogene under the control of the human tissue-specific genetic expression elements.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Stu1 (2876)
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Hpa1 (4333)
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5000
6000

Fig. 1

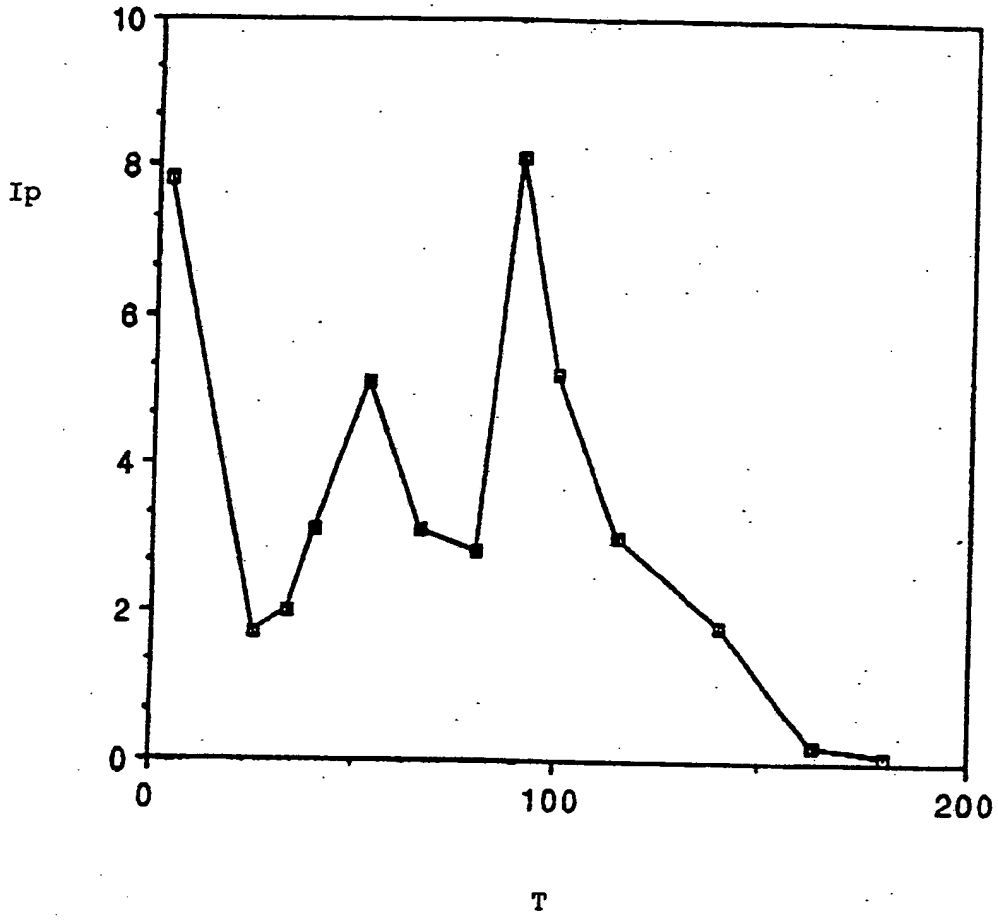

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/02041

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N 5/10 ; C12N 15/85 ; C12P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NATURE. vol. 315, 09 May 1985, LONDON GB pages 115 - 122; Hanahan D.: "Heritable formation of pancreatic B-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes" see the whole document (cited in the application)	1, 5, 8-12, 14-15
X Y	EP,A,298807 (TRANSGENE S.A.) 11 January 1989 see the whole document	1, 8-12, 14-15 2-3, 6-7, 13
X Y	EP,A,307248 (GENENTECH,INC) 15 March 1989 see the whole document	1, 8-12, 14-15 2-3

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 MARCH 1991	22. 04. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	FERNANDEZ Y BRA F.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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Y	WO,A,8603780 (TECHNICLONE RESEARCH PARTNERS I) 03 July 1986 see the whole document	7, 13
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 83, July 1986, WASHINGTON US pages 4794 - 4798; Holt,J.T.et al: "Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation" see the whole document	6
X	EP,A,263908 (BATTELLE MEMORIAL INSTITUTE) 20 April 1988 see the whole document	1, 4, 8-12, 14-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/0204

SA 43081

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EP-A-298807	11-01-89	FR-A-	2637613	13-04-90
		JP-A-	1030580	01-02-89
EP-A-307248	15-03-89	AU-A-	2209988	20-04-89
		JP-A-	1120300	12-05-89
WO-A-8909816	19-10-89	None		
WO-A-8603780	03-07-86	EP-A-	0207147	07-01-87
		JP-T-	62501537	25-06-87
EP-A-263908	20-04-88	AU-A-	8078487	06-05-88
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